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Gardenin B-induced cell death in human leukemia cells involves multiple caspases but is independent of the generation of reactive oxygen species

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ABSTRACT

Flavonoids have attracted great interest due to their possible anticancer activities. Here we investigated the antiproliferative activity of the flavonoids isolated from *Baccharis scandens* against human leukemia cell lines and found that the methoxyflavonoid gardenin B was the most cytotoxic compound against HL-60 and U-937 cells, showing IC_{50} values between 1.6 and 3.0 μM, but had no significant cytotoxic effects against quiescent or proliferating human peripheral blood mononuclear cells. These effects on viability were accompanied by the concentration- and time-dependent appearance of apoptosis as evidenced by DNA fragmentation, formation of apoptotic bodies and a sub-G_1 ratio increase. Comparative studies with the best-studied bioflavonoid quercetin indicate that gardenin B is a more cytotoxic and more apoptotic inducer than quercetin. Cell death induced by gardenin B was associated with: (*i*) a significant induction of caspase-2, -3, -8 and -9 activities; (*ii*) cleavage of the initiator caspases (caspase-2, -8 and -9), of the executioner caspase-3, and of poly(ADP-ribose) polymerase; and (*iii*) a concentration-dependent reactive oxygen species generation. In conclusion, apoptosis induced by gardenin B is associated with activation of both the extrinsic and the intrinsic apoptotic pathways of cell death and occurs through a mechanism that is independent of the generation of reactive oxygen species.

1. Introduction

Molecular target therapies represent a significant advance in the treatment of cancer. However, many of them are highly toxic, very expensive, show limited efficacy and most patients experience relapse after a few disease-free months. Hence there is an urgent need to develop new anticancer strategies. Phytochemicals may be of use because they are better tolerated than synthetic chemotherapeutics, exhibit broader mechanisms of action and show higher affinity against cancer targets [1]. These plant-derived compounds have been shown not only to exert protective effects but also have an impact on the cellular hallmarks of cancer, including, among others, sustained proliferative signaling and resistance to apoptosis [2].

Flavonoids are naturally occurring biologically active polyphenolic compounds that have attracted attention due to their potential anticancer effects. Some mono- or dimethoxylated flavones, in contrast to their nonmethylated analogues, have potent biological activity and antiproliferative effects that are selective for cancer cells, but show low cytotoxic or cytostatic activity in normal cells [3]. Interest in methoxylated flavonoids is motivated by three major properties: (*i*) greater chemopreventive properties than the unmethylated flavonoids; (*ii*) high metabolic stability and (*iii*)

Abbreviations: DHE, dihydroethidium; H_2-DCF-DA, 2′,7′-dichlorodihydrofluorescein diacetate; IC_{50}, 50% inhibition of cell growth; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide; NAC, *N*-acetyl-L-cysteine; PARP, poly(ADP-ribose) polymerase; PI, propidium iodide; ROS, reactive oxygen species.

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potentially increased bioavailability [4–6]. Moreover, the methoxylated flavonoids might pass through biological membranes more easily than the corresponding demethylated (unmethylated), due to their higher lipophilicity, allowing them to reach higher intracellular concentrations. Previous studies have shown that polymethoxyflavonoids such as 5-hydroxy-6,7,8,3′,4′-pentamethoxyflavone and 5-hydroxy-3,6,7,8,3′,4′-hexamethoxyflavone showed strong inhibitory activities against the proliferation and induced apoptosis of the HL-60 cell line [7]. The latter effect, induction of apoptotic cell death, is recognized as a property that is useful for identifying anticancer drugs [8].

Previous studies have shown that *Baccharis scandens* is a source of flavonoids, including methoxylated derivatives. The naturally occurring flavonoid gardenin B has been recently assessed for cytotoxicity against the human colon cancer cell line HCT116 [9] but, so far, its potential use in antileukemia therapy is largely unexplored. The main aim of this work is to determine the potential cytotoxic effects of the polyphenolic compounds isolated from *B. scandens* and the pathways of cell death triggered by the naturally occurring flavonoid gardenin B in two human leukemia cells. HL-60 is an acute myeloid leukemia cell line which was established from the peripheral blood of a 35-year-old woman with acute myeloid leukemia (AML FAB M2) in 1976 [10]. The U-937 is a promonocytic, human myeloid leukemia cell line which was isolated from a histiocytic lymphoma of a 37 year old male [11]. These cell lines are used in biomedical research and are important tools in the investigation of cytotoxic compounds as well as in the exploration of the signal transduction pathways of cell death. We have also specifically evaluated the activation of the caspase cascade and the generation of reactive oxygen species.

2. Materials and methods

2.1. Reagents

Baccharis scandens Ruiz & Pav Pers. ("Chilca dulce") was collected in La Rioja Province, Argentine. The plant was identified by Dr. L. Ariza-Espinar, Universidad Nacional de Córdoba, Argentina (Voucher: Ariza Espinar, L. 7432, Prov. La Rioja, Depto. Vichincha, Laguna Brava). Dried aerial parts (1200 g) of *B. scandens* were extracted with acetone at room temperature (×3). The organic extract was subjected to flash chromatography on silica gel, eluting with *n*-hexane-EtOAc gradient to afford 10 fractions. Each fraction obtained was monitored by thin layer chromatography (C_6H_6–dioxane–AcOH 30:5:1). The analysis of the polar fractions indicated a mixture of flavonoids, which were separated and purified by Sephadex LH-20 column chromatography, eluting with MeOH to furnish the flavonoids: salvigenin (87 mg), gardenin B (63 mg), xanthomicrol (50 mg) and quercetin (38 mg). Structural identities of these compounds were determined spectroscopically (proton nuclear magnetic resonance and ^{13}C nuclear magnetic resonance, infrared and UV/Visible spectroscopy and mass spectrometry) as described previously [12]. Purity of these compounds was 99.0% as judged by high-performance liquid chromatography. Stock solutions of 10 mM flavonoids were made in dimethyl sulfoxide (DMSO) and aliquots were frozen at −20 °C. The following antibodies were used according to the manufacturer's instructions: Bax, rabbit polyclonal; poly(ADP-ribose) polymerase (PARP), mouse monoclonal; caspase-2, mouse monoclonal; cytochrome *c*, mouse monoclonal (BD PharMingen, San Diego, CA, USA); caspase-3, rabbit polyclonal (Assay Designs, Ann Arbor, MI, USA); caspase-8 and caspase-9, mouse monoclonal (Enzo Life Sciences, Farmingdale, NY, USA); Bcl-2, mouse monoclonal (Santa Cruz Biotechnology, Santa Cruz, CA, USA); β-actin, mouse monoclonal (Sigma, Saint Louis, MO, USA). Secondary antibodies were from GE Healthcare Bio-Sciences AB (Little Chalfont, UK). All other chemicals were obtained from Sigma (Saint Louis, MO, USA).

2.2. Cell culture and cytotoxic assays

The human leukemia HL-60 and U-937 cells were obtained from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany) and cultured in RPMI 1640 containing 2 mM L-glutamine supplemented with 10% (v/v) heat-inactivated fetal bovine serum as previously described [13]. Human peripheral blood mononuclear cells (PBMCs) were isolated from heparin-anticoagulated blood of healthy volunteers by centrifugation with Ficoll-Paque Plus (GE Healthcare Bio-Sciences AB; Uppsala, Sweden). PBMCs were also stimulated with phytohemagglutinine (PHA, 2 μg/ml) for 48 h before experimental treatment. The cytotoxicity of flavonoids on human tumor and human PBMC cells was analyzed by colorimetric MTT assay as previously described [13]. Concentrations inducing a 50% inhibition of cell growth (IC_{50}) were determined graphically using the curve fitting algorithm of the computer software Prism™ 4.0 (GraphPad). In all cytotoxicity assays etoposide was included as a positive control.

2.3. Evaluation of apoptosis

The rate of apoptotic cell death was determined by flow cytometric analysis of propidium iodide-stained nuclei and by using an annexin V-FITC apoptosis detection kit (BD Pharmingen) according to the manufacturer's protocol. Fluorescent microscopy was also used to identify nuclear changes associated with apoptosis, together with DNA laddering on agarose gel electrophoresis.

2.4. Western blot analysis

Immunoblot analysis of caspases, Bax, Bcl-2, cytochrome *c* and PARP was performed as previously described [13].

2.5. Assay of caspase activity

Caspase activity was determined by measuring proteolytic cleavage of the chromogenic substrates VDVAD-*p*NA (for caspase-2 activity), DEVD-*p*NA (for caspase-3 like protease activity), IETD-*p*NA (for caspase-8 activity) and LEHD-*p*NA (for caspase-9 activity), as described previously [13].

2.6. In vitro tubulin polymerization assay

In vitro tubulin polymerization assays were performed as described by the manufacturer (Cytoskeleton, Inc., Denver, CO). Briefly, gardenin B was incubated with purified bovine tubulin in 80 mM PIPES buffer (pH 7.0) containing 1 mM GTP, 1 mM EGTA, 1 mM $MgCl_2$, and 10% glycerol, and the increase in absorbance was measured at 340 nm in a Beckman Coulter DTX880 microplate reader at 37 °C and recorded every 30 s for 50 min. Taxol (10 μM) and colchicine (5 μM) were used as positive controls of promotion and inhibition of tubulin polymerization, respectively.

2.7. Reactive oxygen species determination

The intracellular generation of peroxides and superoxide was monitored by flow cytometry with the probes 2′,7′-dichlorodihydrofluorescein diacetate (H_2-DCF-DA) and dihydroethidium (DHE), respectively. Cells were treated with or without gardenin B and H_2-DCF-DA (2 μM) or DHE (2 μM) was added to the medium 30 min before the end of incubation with gardenin B. Cells were irradiated with an argon laser at 488 nm, and fluorescence was

detected at 525 nm (DCF) and 568 nm (DHE) in the flow cytometer.

2.8. Analysis of the mitochondrial membrane potential $\Delta\Psi_m$

Cells were treated with gardenin B (3 μM) for different time periods and incubated with the fluorescent probe 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide (JC-1, 10 μM) for the last 30 min and flow cytometric analysis was carried out using a BD FACSVerse™ cytometer (BD Biosciences, San Jose, CA, USA). As a positive control, cells were treated with 50 μM of the protonophore CCCP (carbonyl cyanide *m*-chlorophenylhydrazone).

2.9. Statistical analysis

Statistical significances of differences between means of control and treated samples were determined using Student's *t*-test. *P* values of <0.05 were considered significant.

3. Results

3.1. Gardenin B inhibits cell viability of human leukemia cell lines

In the present study, the potential cytotoxic properties of the naturally occurring flavonoids isolated from the plant *Baccharis scandens* were evaluated in human leukemia cells (Fig. 1).

Gardenin B was the most cytotoxic compound for human leukemia cells with an IC_{50} value (the concentration that induces a 50% inhibition of cell growth) of 1.6 ± 0.7 μM and 3.0 ± 1.5 μM in HL-60 and U-937 cells, respectively (Table 1). The antitumor agent etoposide was used as a positive control for both HL-60 (IC_{50} = 0.5 ± 0.1 μM) and U-937 cells (IC_{50} = 1.5 ± 0.3 μM). The methoxy group located at C-8 is essential in conferring cytotoxicity since gardenin B exhibited a higher potency than salvigenin (5-hydroxy-6,7,4′-trimethoxyflavone). Moreover, the methoxy group located at the C-4′position seems to be essential as well, since the methylation of the hydroxyl group at position C-4′of xanthomicrol (5,4′-dihydroxy-6,7,8-trimethoxyflavone) to produce gardenin B enhanced the potency against cell growth inhibition. It thus seems that both C-8 and C-4′methoxy groups are the major determinants of cytotoxicity. Although the polyhydroxylated flavonoid quercetin showed a similar potency to salvigenin against HL-60 cells, it was at least 5-fold less potent than salvigenin against U-937 cells and it displayed lower cytotoxicity than gardenin B in both cell lines (Table 1).

Table 1
Effects of flavonoids isolated from *B. scandens* on the growth of the human leukemia cell lines.

Compound	IC_{50} (μM)	
	HL-60	U-937
Salvigenin	47.1 ± 9.2	10.5 ± 0.3
Gardenin B	1.6 ± 0.7	3.0 ± 1.5
Xanthomicrol	33.9 ± 3.0	15.5 ± 3.1
Quercetin	45.2 ± 5.0	50.9 ± 5.0

Cells were cultured for 72 h and the IC_{50} values were calculated as described in the Experimental Section. The data shown represent the means ± SEM of 3–5 independent experiments with three determinations in each.

Treatment with the methoxyflavonoid gardenin B (5-hydroxy-6,7,8,4′-tetramethoxyflavone) resulted in a concentration-dependent inhibition of cell viability and induced a significant reduction in the number of cells (Fig. 2A and B). Quiescent and proliferating PBMCs were more resistant than both HL-60 and U-937 cells, even at 30 μM of gardenin B (Fig. 2C).

Since gardenin B was the most cytotoxic methoxyflavonoid against human leukemia cells, further experiments were performed with this compound. To determine the mechanism involved in gardenin B-induced cytotoxicity, DNA fragmentation, fluorescent microscopy and flow cytometry experiments were done. As shown (Fig. 3A), DNA fragmentation (a hallmark of apoptosis) markedly increased in gardenin B-treated cells. The fluorescent microscopy experiments using Hoescht 33258 revealed increases in condensed and fragmented chromatin which is typical of apoptotic cells (Fig. 3B). Evaluation of the percentage of sub-G_1 (hypodiploid) cells by flow cytometry showed that the percentage of apoptotic cells increased approximately 7-fold in gardenin B-treated HL-60 compared with control cells (37.7 ± 3.5% vs 5.3 ± 0.9%) after 24 h exposure at a concentration as low as 3 μM (Table 2 and Fig. 3C). However, the percentage of hypodiploid cells increased about twofold in quercetin-treated HL-60 cells but at a concentration

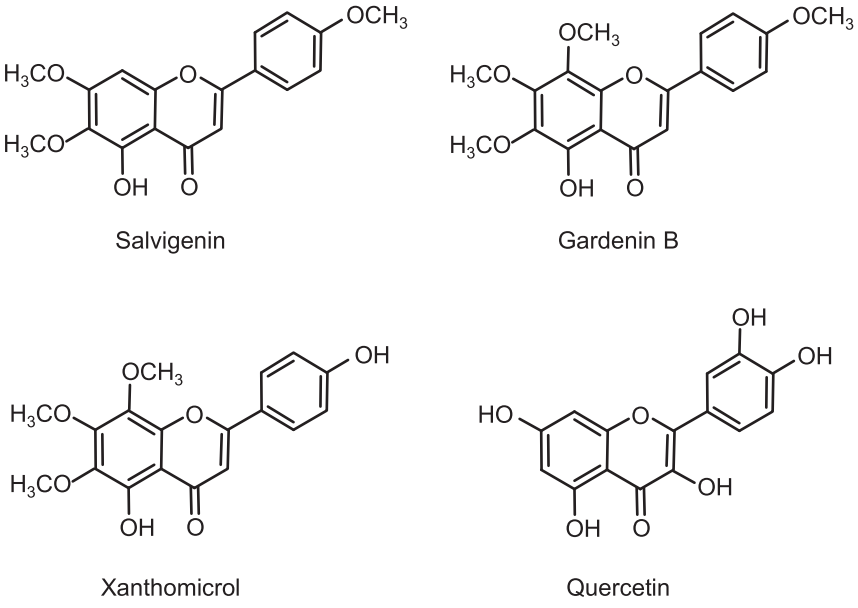

Fig. 1. Chemical structures of the flavonoids isolated from *B. scandens*.

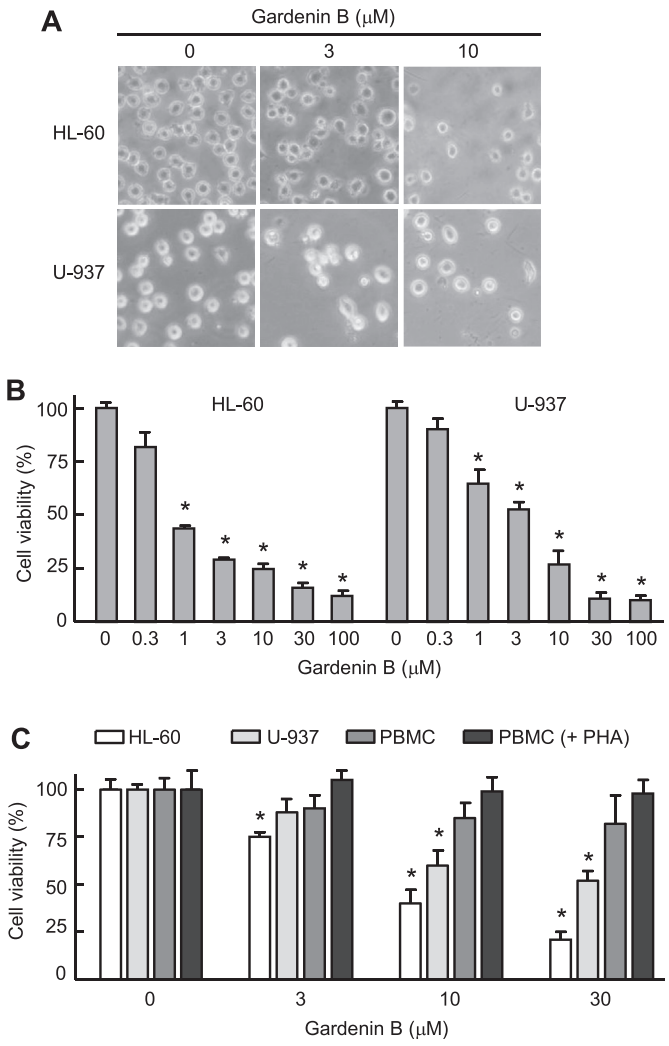

Fig. 2. Effect of gardenin B on human leukemia cells viability. (A) Cells were incubated with vehicle (control) or the indicated concentrations of gardenin B for 24 h and images were obtained with an inverted phase-contrast microscope. (B) Effect of gardenin B on cell viability. Cells were cultured in the presence of the indicated concentrations of gardenin B for 72 h, and thereafter cell viability was determined by the MTT assay. (C) Differential effect of gardenin B on cell viability of normal peripheral blood mononuclear cells (PBMCs) versus HL-60 and U-937 cells. Human leukemia, and quiescent and phytohemagglutinine-activated PBMC cells from healthy human origin were cultured in the presence of the specified concentrations of gardenin B for 24 h. Values represent means ± SE of three independent experiments each performed in triplicate. $^{*}P < 0.05$, significantly different from the corresponding control.

tenfold higher (results not shown). Moreover, gardenin B treatment (3 μM) also led to the exposure of phosphatidylserine on the outside of the plasma membrane as detected by annexin V-FITC staining in HL-60 cells (Fig. 3D).

To study the effects of gardenin B on microtubule formation, we used an *in vitro* tubulin polymerization assay and monitored the increase in absorbance of the reaction mixture. The results indicate that gardenin B did not inhibit tubulin polymerization at any concentration assayed. Colchicine and taxol were used as positive controls of inhibition and promotion of tubulin polymerization, respectively (Fig. 3E).

To determine whether gardenin B-induced cytotoxicity involves alterations in cell cycle progression, flow cytometric analyses were included in this study. As shown in Table 2, gardenin B caused a significant S and G_2-M arrest at the expense of the G_1 phase cell population at 6 h of treatment in a dose-dependent manner. The percentage of control-treated cells in G_2-M phase was ~20%, which increased to ~30% after treatment with gardenin B for 6 h. A similar trend in G_2-M arrest was observed after 12 h of treatment.

There was also a decrease in the percentage of cells in G_1 phase which was accompanied by an increase in G_2-M phase cell population at 12 h of treatment, but this effect invariably starting diminishing with the increase in treatment time (24–48 h) in HL-60 cells. Moreover, the percentage of hypodiploid cells (i.e. sub-G_1 fraction) increased about 5-fold, 7-fold and 10-fold in gardenin B-treated HL-60 compared with control cells after 12, 24 and 48 h exposure, respectively (Table 2).

3.2. Effects of gardenin B on mitochondrial cytochrome c release and on caspases- and PARP-processing

To determine whether gardenin B-triggered apoptosis involves the activation of caspases, HL-60 and U-937 cells were treated with increasing concentrations of this flavonoid for 24 h, and initiator (caspase-8 and -9) and executioner (caspase-3) caspases were determined by Western blot. As shown in Fig. 4A, gardenin B stimulated the cleavage of pro-caspase-2, -3, -8 and -9 and poly(ADP-ribose)polymerase (PARP). Apoptosis induction occurs via the intrinsic and extrinsic pathways since gardenin B stimulated the cleavage of inactive pro-caspase-9 to the active 35–37 kDa fragments and significantly promoted procaspase-8 hydrolysis, which was detected as a decrease (Fig. 4A). This methoxyflavonoid also induced pro-caspase-2 cleavage as visualized as a reduction in the proenzyme. To determine the role of cytochrome *c* on gardenin B-induced apoptosis cytosolic preparations were analyzed by immunoblotting. As demonstrated (Fig. 4A), a significant increase in cytochrome *c* in the cytosol was already detected with 3 μM gardenin B after 24 h. We also investigated the expression of the Bcl-2 family members which control the intrinsic apoptotic pathway. As shown in Fig. 4A, gardenin B decreased the Bcl-2 levels in both cell lines in a concentration dependent manner, although they were more pronounced in U-937 cells, while there were no changes in Bax levels.

As processing does not always correlate with activity, enzymatic activities of caspase-2, caspase-3-like proteases (caspase-3/7), caspase-8 and caspase-9 were also investigated in HL-60 and U-937 cells. Cell lysates were assayed for cleavage of the tetrapeptides VDVAD-*p*NA, DEVD-*p*NA, IETD-*p*NA and LEHD-*p*NA as specific substrates for caspase-2, caspase-3/7, caspase-8 and caspase-9, respectively. As shown (Fig. 4B), a dose-response dependent induction of these caspases was observed after 24 h of treatment. In HL-60 cells, caspase-2 and caspase-3/7 activities increased 2-fold in response to gardenin B, while caspases-8 and -9 activities increased 1.7-fold over the control, respectively. Similar results were obtained in U-937 cells, although caspase-3/7 activity increased 3-fold over the control and the activation of caspase-8 was higher than caspase-9 activation.

To explore whether the release of cytochrome *c* is associated with mitochondrial membrane potential dissipation, HL-60 and U-937 cells were treated with gardenin B (3 μM) for different times (12–24 h), stained with JC-1 and analyzed by flow cytometry. Our results show that $\Delta\Psi_m$ remained intact for at least 24 h of treatment, which suggests that the disruption of the mitochondrial membrane potential is not involved in the apoptosis induced by gardenin B. In these experiments, the protonophore CCCP was used as a positive control (Fig. 4C).

3.3. Gardenin B increases intracellular ROS levels

Generation of intracellular reactive oxygen species (ROS) is considered one of the key mediators of apoptotic signaling for

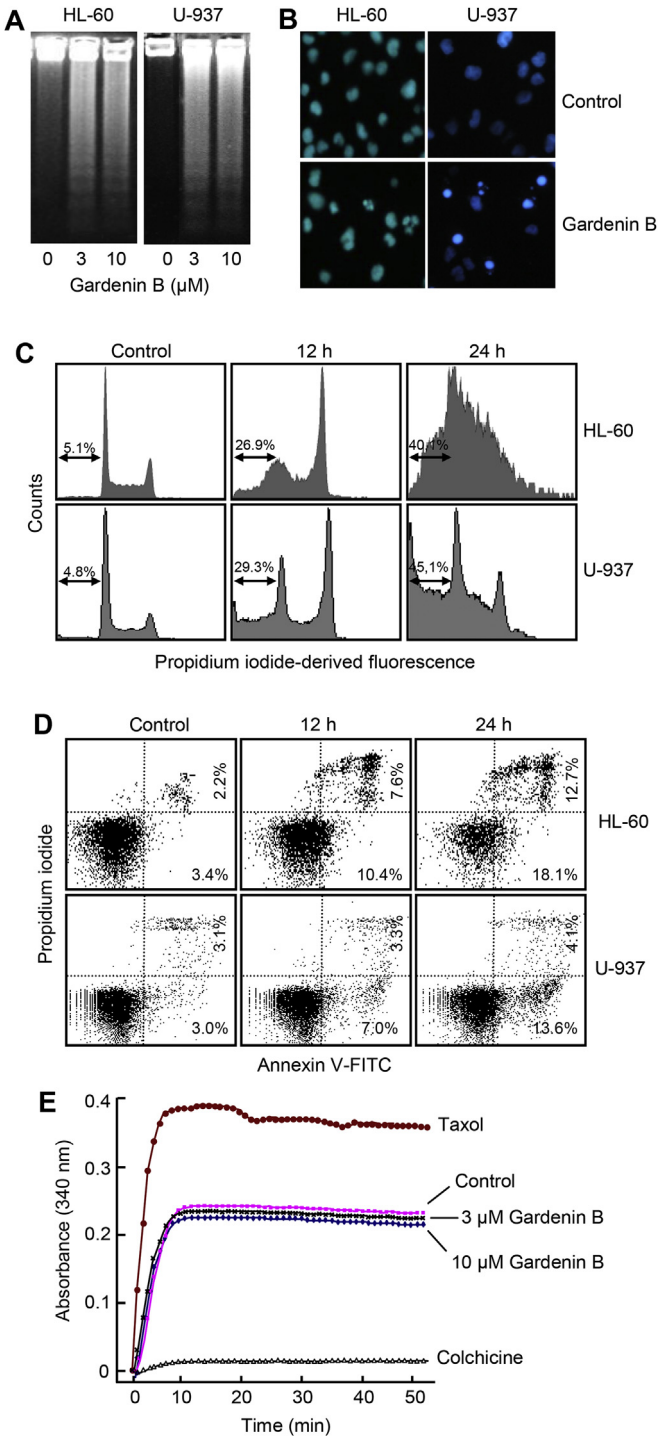

Fig. 3. Effects of gardenin B on apoptosis induction in HL-60 and U-937 human myeloid leukemia cells. (A) Qualitative assessment of apoptotic DNA damage. Cells were treated with the indicated concentrations of gardenin B and genomic DNA was extracted, separated on an agarose gel and visualized under UV light by ethidium bromide staining. (B) Photomicrographs of representative fields of human leukemia cells stained with bisbenzimide trihydrochloride to evaluate nuclear chromatin condensation (i.e. apoptosis) after treatment with 3 μM gardenin B for 24 h. (C) Cells were incubated in the presence of gardenin B (3 μM) and harvested at the indicated times and subjected to DNA flow cytometry using propidium iodide labeling. Hypodiploid cells (apoptotic cells) are shown in region marked with an arrow. (D) Flow cytometry analysis of annexin V-FITC and propidium iodide-stained cells after treatment with 3 μM gardenin B for the indicated times. Cells appearing in the lower right quadrant show positive annexin V-FITC staining, which indicates phosphatidylserine translocation to the cell surface, and negative propidium iodide (PI) staining, which demonstrates intact cell membranes, both features of early apoptosis. Cells in the top right quadrant are double positive for annexin V-FITC and PI and are undergoing late apoptosis. Data are representative of three separate experiments. (E) Gardenin does not affect the tubulin polymerization *in vitro*. Purified tubulin protein in a reaction buffer was incubated at 37 °C in the absence (control) or in the presence of taxol (10 μM), colchicine (5 μM) or the indicated concentrations of gardenin B and the absorbance at 340 nm was measured in a microplate reader.

many antitumoral agents [14]. Therefore, the redox status of HL-60 cells treated with gardenin B was monitored by the oxidation-sensitive fluorescent dye 2′,7′-dichlorodihydrofluorescein diacetate (H_2-DCF-DA). A dose- and time-dependent increase in DCF fluorescence was detected in gardenin B-treated cells (Fig. 5A and B). A fast generation of ROS was detected at 2 h after treatment although the highest levels (>2-fold increase compared with control) were not reached until 10 h (Fig. 5B). For comparison, exposure to H_2O_2 (100 μM) was associated with a marked increase in ROS levels (results not shown). To determine whether gardenin B induces superoxide anion, cells were incubated with dihydroethidium (DHE) which is relatively specific to this free radical and reacts only minimally to hydrogen peroxide. As shown in Fig. 5C a slight increase in DHE-derived fluorescence in HL-60 cells was detected at longer exposure time.

To determine whether the generation of ROS is involved in cell death induced by gardenin B the effects of the antioxidants *N*-acetyl-L-cysteine (NAC, 10 mM), a thiol-containing antioxidant that is capable of directly inactivating ROS and of inducing the production of glutathione [15], were investigated. This antioxidant did not block cell death as assessed by flow cytometry and by the trypan blue exclusion method indicating that gardenin B-induced cell death is independent of ROS production (Fig. 5D and E). Similar results were obtained with the antioxidants tiron (80 μM) and trolox (2 mM) (results not shown).

4. Discussion

Previous studies have shown that different analogs of polymethoxyflavonoids are cytotoxic to tumor cells [16–21]. However, little is known about the mechanism by which potent methoxy flavonoids exhibit their anticancer effects. In this study, we investigated the potential cytotoxic properties of the flavonoids isolated from *B. scandens* against two human leukemia cell lines, HL-60 and U-937. The antiproliferative studies indicate that gardenin B was the most cytotoxic compound against both cell lines. Interestingly, dose-response studies revealed that quiescent PBMC and proliferating PBMC were resistant toward gardenin B. This flavonoid was at least 28-fold and 17-fold more cytotoxic than quercetin against HL-60 and U-937 cells, respectively. Comparing the cytotoxic effect of 5-hydroxy-6,7,4′-trimethoxyflavone and 5-hydroxy-6,7,8,4′-tetramethoxyflavone against human leukemia cells it is clear that the presence of the methoxy group at C-8 is crucial in determining cytotoxicity. And addition, comparison of the effects of 5,4′-dihydroxy-6,7,8-trimethoxyflavone and 5-hydroxy-6,7,8,4′-tetramethoxyflavone on the presence of a methoxy group at 4′ suggests a key role in conferring cytotoxicity. Although the exact target and binding sites of gardenin B have not yet been determined, the introduction of additional methyl groups at C-8 and at C-4′ could facilitate penetration through the cell membrane and increase the cytotoxicity *in vitro*. Different metabolic pathways in cells and differences in compound stability in tissue culture medium might also affect cytotoxicity. The cytotoxic effects were accompanied by the concentration- and time-dependent appearance of apoptosis as determined by DNA fragmentation, apoptotic bodies visualization and sub-G_1 ratio. Comparative studies with the best-studied bioflavonoid quercetin indicate that gardenin B is more cytotoxic and

Table 2
Effect of different durations of treatment with gardenin B on cell cycle phase distribution of HL-60 cells.

		% Sub-G_1	%G_1	%S	%G_2-M
6 h	Control	4.9 ± 0.8	36.8 ± 0.5	36.7 ± 0.8	20.5 ± 1.0*
	3 μM Gardenin B	12.1 ± 2.6*	13.9 ± 1.0*	43.8 ± 0.4*	28.2 ± 1.7*
	10 μM Gardenin B	10.5 ± 1.4*	12.2 ± 0.1*	45.0 ± 1.2*	30.6 ± 1.6*
12 h	Control	5.9 ± 1.2	43.8 ± 1.3*	35.1 ± 1.8	14.2 ± 1.4*
	3 μM Gardenin B	26.1 ± 4.3*	14.7 ± 1.0*	33.1 ± 2.4	24.0 ± 2.9*
	10 μM Gardenin B	25.3 ± 4.6*	12.3 ± 1.2*	34.1 ± 1.6	26.0 ± 3.9*
24 h	Control	5.3 ± 0.9	41.6 ± 8.4*	37.0 ± 5.8	12.9 ± 0.2*
	3 μM Gardenin B	37.7 ± 3.5*	15.6 ± 1.5*	32.1 ± 2.1	6.2 ± 0.1*
	10 μM Gardenin B	36.8 ± 0.6*	13.5 ± 0.8*	34.9 ± 0.1	5.6 ± 0.2*
48 h	Control	4.9 ± 0.8	46.6 ± 2.6*	31.9 ± 2.5	15.6 ± 1.0*
	3 μM Gardenin B	49.3 ± 0.8*	12.9 ± 1.7*	27.8 ± 1.0	5.2 ± 0.4*
	10 μM Gardenin B	53.4 ± 1.4*	9.7 ± 0.9*	26.2 ± 1.1	5.2 ± 0.4*

Cells were cultured with gardenin B for the indicated period of times and the cell cycle phase distribution was determined by flow cytometry. The values are means ± S.E. of three independent experiments with three determinations in each. Asterisks indicate a significant difference ($P < 0.05$) compared with the corresponding controls.

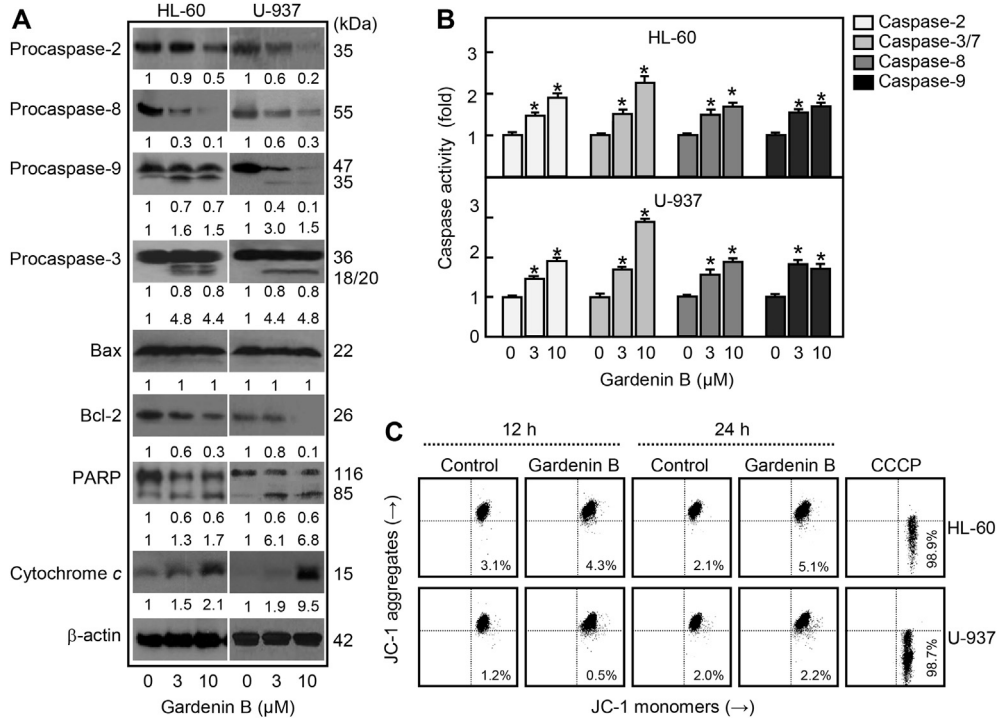

Fig. 4. Involvement of caspases in the induction of apoptosis triggered by gardenin B in human myeloid leukemia cells. (A) Cells were incubated with the indicated concentrations of gardenin B for 24 h and cell lysates or cytosolic extracts were assayed by immunoblotting for the cleavage of procaspases, poly(ADP-ribose) polymerase (PARP), Bax, Bcl-2 and cytochrome *c* release, respectively. Numbers below each panel indicate fold differences after normalization to β-actin. (B) Activation of caspases in response to gardenin B. Cells were treated as above, and cell lysates were assayed for caspase-2, -3/7, -8 and -9 activities. Results are expressed as *n*-fold increases in caspase activity compared with the control. Values represent the means ± SEs of three independent experiments each performed in triplicate. $^*p < 0.05$, indicates a significant difference from the untreated control. (C) Cells were treated with the gardenin B (3 μM) for the indicated times, harvested and $\Delta\Psi_m$ analyzed by flow cytometry after staining with the JC-1 probe. Similar results were obtained in two separate experiments each performed in triplicate. As a positive control, aliquots of cells were stained in the presence of 50 μM CCCP.

more apoptotic inducer than quercetin. Cell cycle analysis performed in HL-60 cells showed that inhibition of cell viability by gardenin B was caused by a significant cell cycle arrest at the S and G_2-M phases and accompanied by an increase in sub-G_1 fraction and phosphatidylserine externalization, indicating apoptotic cell death. This methoxyflavonoid promotes the formation of apoptotic bodies and the internucleosomal degradation of DNA, resulting in the formation and eventual release of oligonucleosomal DNA fragments.

The arrest of cells in the G_2-M phase of the cell cycle induced by gardenin B might be explained by the inhibition of microtubule formation or by changes in the expression and/or activity of G_2-M cell cycle regulators. Many cytotoxic drugs induce G_2-M arrest by targeting microtubules [22] and some flavonoids have been shown to perturb microtubules polymerization via tubulin binding [23–26]. To investigate whether gardenin B directly targets tubulin, it was performed an *in vitro* tubulin polymerization assay with increasing concentrations of gardenin B and observed that this methoxyflavonoid did not have any apparent effect on the tubulin polymerization. In contrast, colchicine and taxol inhibited and promoted the tubulin polymerization, respectively. However, this does not exclude the possibility that gardenin B metabolites may bind tubulin inside the cells. Future studies will be necessary to determine the effect of gardenin B on G_2-M cell cycle regulators such as the cyclin-dependent kinase-1, cyclin-dependent kinase inhibitor $p21^{Cip1}$, B-type cyclin isoforms and cdc25C.

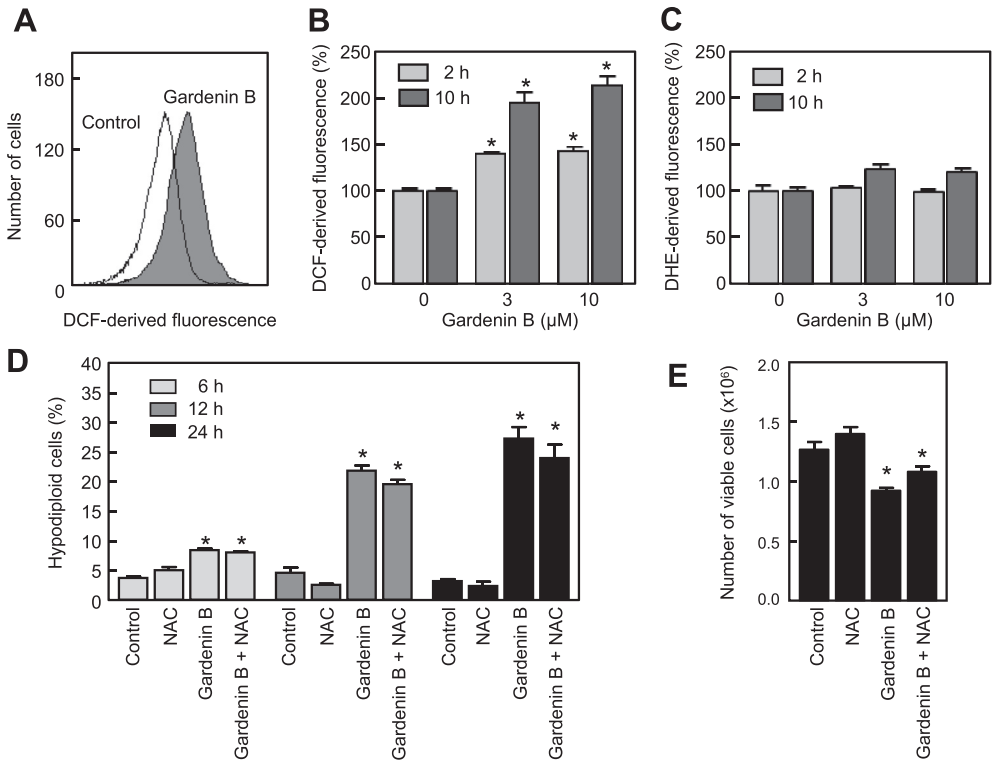

Fig. 5. Gardenin B increases ROS generation in HL-60 cells. (A) Cells were treated with gardenin B for 2 h and the fluorescence of oxidized H_2DCF was determined by flow cytometry. Similar results were obtained from three independent experiments. Cells were incubated in the presence of the indicated concentrations of gardenin B for the indicated time points and the fluorescence of oxidized H_2DCF (B) or DHE (C) was determined by flow cytometry. (D) Lack of apoptosis inhibition by *N*-acetyl-L-cysteine (NAC, 10 mM) in gardenin B-treated cells. Cells were preincubated with NAC for 1 h and then treated in the absence or the presence of gardenin B for different times. Apoptosis was quantified by flow cytometry after propidium iodide staining. (E) Cells were preincubated with NAC for 1 h and then incubated with gardenin B for 24 h and thereafter cell viability was determined by the trypan blue exclusion method. $^*P < 0.05$ indicates a significant difference from the untreated control.

Previous studies have shown that monodemethylated pentamethoxyflavones were much more potent apoptotic inducers than their permethoxylated counterparts [19]. However, little is known about the caspase activation triggered by gardenin B. Our study demonstrates that gardenin B-induced cell death was associated with significant induction of caspase-2, caspase-3, caspase-8 and caspase-9 activities. Although the protease specificities of human caspase-2, caspase-3 and caspase-7 largely overlap, with a DEVD↓G consensus cleavage sequence [27], the specific tetrapeptide substrate for caspase-2 VDVAD-*p*NA was effectively hydrolyzed. Caspase-2 has been implicated in apoptotic cell death induced by multiple intrinsic and extrinsic stimuli including DNA damage, reactive oxygen species and cytoskeletal disruption [28], as well as in the regulation of the G_2-M checkpoint [29,30].

In accordance with the caspase activation experiments, this flavonoid also induced the cleavage of both initiator caspases (caspase-8 and -9) and the executioner caspase-3. The DNA repair enzyme and known caspase-3 substrate poly(ADP-ribose) polymerase was also hydrolyzed to the 85 kDa fragment by gardenin B. The fact that caspase-9 is activated by gardenin B suggests that cell death is linked with cytochrome *c* release. Gardenin B also down-regulates the expression of the anti-apoptotic Bcl-2 but does not modulate the expression of Bax. Nonetheless, these results do not exclude the possible involvement of a receptor-mediated mechanism since gardenin B also induces caspase-8 activation which is essential for the apoptotic response downstream of death receptors. It would be interesting to investigate in the future the effect of gardenin B on expression and/or activation of death receptors, including Fas, tumor necrosis factor 1 (TNFR1) and tumor necrosis factor-related apoptosis-induced ligand (TRAIL) receptors, death receptor 4 (DR4) and death receptor 5 (DR5). These receptors belong to the death receptor subgroup in the tumor necrosis factor (TNF) receptor superfamily. TRAIL interaction with DR4 or DR5 initiates a caspase-driven apoptotic pathway upon formation of a Death-Inducing Signaling Complex similar to that induced by Fas-Ligand/Fas interaction. Several natural products (including flavonoids) and also synthetic derivatives have been shown to induce apoptosis through the expression of DR4 and DR5 and might be useful in the treatment of TRAIL-resistant tumor cells alone or in combination with TRAIL [31,32].

Several researchers, including ourselves, have described that some flavonoid derivatives induce apoptosis through reactive oxygen species production [33,34]. For example, astragalin heptaacetate-, tamarixetin- and eupatorin-induced cell death was dependent on ROS generation [13,33,35]. Apoptosis was induced by 5-hydroxy-3,6,7,8,3′,4′-hexamethoxyflavone through reactive oxygen species production that decreased mitochondrial *trans*-membrane potential in human leukemia cells [34]. Although the antiproliferative effect of gardenin B is associated with an increase in the intracellular level of ROS, this did not seem to play a pivotal role in the apoptotic process since the antioxidants trolox, tiron and *N*-acetyl-L-cysteine were unable to block cell death. Similar results were previously obtained for the flavonoid derivative trifolin acetate which induces ROS, but this is not necessary to trigger cell death [36]. Nevertheless, the IC_{50} values for gardenin B were lower than for trifolin acetate in HL-60 and U-937. In conclusion, gardenin B is cytotoxic against human leukemia cells, blocks proliferation by arresting the cells in the G_2-M phase and is an apoptotic inducer. The cell death triggered by this methoxyflavonoid was associated with activation of both the extrinsic and the intrinsic apoptotic pathways.

Conflict of interest

We declare no conflict of interest.

Acknowledgments

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